CHROMATOGRAPHY OF CATALASE AND CARBONIC ANHYDRASE ACTIVITY FROM CRUDE RED BLOOD CELL HEMOLYSATES ON DIETHYLAMINOETHYL CELLULOSE

by

Homer Alan Lozeron

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CHROMATOGRAPHY OF CATALASE AND CARBONIC ANHYDRASE ACTIVITY FROM CRUDE RED BLOOD CELL HEMOLYSATES ON DIETHYLAMINOETHYL CELLULOSE

A DISSERTATION
SUBMITTED TO THE FACULTY OF GRADUATE STUDIES
IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE
OF MASTER OF SCIENCE

DEPARTMENT OF BIOCHEMISTRY
by
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EDMONTON, ALBERTA
APRIL, 1959.

ABSTRACT

An attempt has been made to resolve carbonic anhydrase and catalase activities selectively from crude red blood cell hemolysates of hog blood on the diethylaminoethyl-cellulose ion-exchange resin of Sober and Peterson.

At low ionic strengths (0.0025 - 0.0050M phosphate buffer) under a variety of pH conditions (6.60 - 7.60), in which hemoglobin (95% of the total protein) was not bound to the resin to any large extent, only a portion of the carbonic anhydrase activity (10%) could be selectively adsorbed on and eluted from the resin. A 95-fold increase in specific activity had been attained, however.

Under the above conditions, catalase activity was quantitatively adsorbed. The hemoglobin and carbonic anhydrase activity could be completely eluted from the resin before the quantitative elution, via an ionic gradient, of catalase. This high yield of catalase having a Katalase - fahigkeit of 23,000 suggested that a significant purification had taken place.

ACKNOWLEGEMENTS

I wish to express my sincere appreciation to Dr. H. B. Collier for his assistance and guidance throughout the course of this work. I also wish to thank other members of the department for their interest, Mr. P. Beamont and Mr. R. Clelland for experimental materials and apparatus and Mr. R. Schaapman, who so kindly delivered blood and dry ice.

The writer also gratefully acknowledges the financial assistance granted by the Defence Research Board of Canada, grant.no. 9350-06.

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INTRODUCTION

Enzymic studies of the red blood cell have been generally carried out on crude cell hemolysates or on purified extracts obtained after ethanol-chloroform precipitation of the hemoglobin, provided the enzyme was reasonably stable to this treatment. The classical ammonium sulfate ($(NH_4)_2SO_4$) fractionation, selective precipitation with organic solvents, or selective adsorption on phosphate or alumina gels (1)(2) were then generally applied in order to attain more extensive purification.

The primary problem in the purification of any enzyme from the hemolysate involved the separation of the enzyme from the bulk of the hemoglobin (Hb). If the enzymic component was found to be extremely labile to the ethanol-chloroform treatment, removal of the Hb presented a major problem. With the exception of horse Hb (3), salt fractionations of the hemoglobin with $(NH_4)_2SO_4$ did not prove feasible because it did not selectively precipitate. Recently, however, acid phosphatase has been separated from the bulk of the Hb by selective adsorption and elution (batchwise) on specially prepared calcium phosphate gels (4).

Although the various hemoglobins have been quite extensively studied on Amberlite (IRC-50) (5)(6), it does not appear that the resolution of any of the enzymic components has been investigated. Only one reference has been cited in which the resin Dowex-2 has been applied to a hemolysate to resolve acid phosphatase from the hemolysate (7).

In general, the chromatographic resolution of proteins has not met with the same general success as that attained with amino acids and other smaller molecules, because of various special problems involved. To the present time, achievements using paper chromatography have been made in only limited directions (8). Protein mixtures have generally given spots which were neither reproducible nor discrete with various degrees of tailing (9). Up to the present time no general method has been worked out.

The liquid-liquid chromatographic method has had only limited success for several proteins (10)(11) due to a variety of reasons. First, the choice of a solvent system in which the organic component does not denature the protein but permits proper partition of the protein must be considered. The protein must be resistant to surface inactivation, and the sensitivity of the partition to temperature may have to be considered (11). Consequently, it did not appear that this system could be used to resolve a number of enzymic proteins at one time.

With the advent within the last few years of a new method of ion-exchange chromatography for the resolution of many proteins, it seemed worthwhile to try this technique as a more general approach to enzymic fractionation.

The choice of a particular resin had to be considered. The resin, IRC-50, has found application for the resolution of the smaller, basic proteins (12)(13), fragile, neutral proteins (5)(6) and for studies of enzyme microheterogeneity of these systems (14). It has been found to be limited, however, with respect to protein capacity with low yields in specific instances (9).

The behavior of a partially purified extract of catalase on this resin will now be discussed in order to indicate specific limitations of this particular system when applied to resolve "acidic" proteins, and to show how the chromatographic method finally chosen would in part alleviate these limiting factors.

Catalase extract was obtained by centrifuging red blood cells from fresh, oxalated hog blood, washing three times with saline, hemolyzing and treating with ethanol-chloroform according to Huennekens et al. (2). This extract was applied to a column of XE-64 (finely powdered IRC-50) purified according to Moore and Stein (15). Catalase activity and protein were determined as described under "Experimental".

With 0.10M phosphate buffer, at pH values below 6.2, catalase was completely adsorbed and could not be eluted by increasing the ionic strength with NaCl. The adsorption at pH 6.2 was probably due to the presence of secondary forces (H-bonding between resin and protein) since catalase would possess a negative charge.

At a pH value of 6.50 (0.10M) however, catalase could be completely separated from the Hb. Catalase activity passed through the column unretarded, but the residual Hb remaining after the ethanol-chloroform fractionation appeared as a narrow band bound tightly to the top of the column. This system was not studied further for a variety of reasons.

From these preliminary experiments it was evident that certain modifications would be necessary if a more general chromatographic method were to be developed, especially enzymic proteins extremely labile to ethanol-chloroform fractionation (even catalase activity was destroyed to the extent of 50-60%). The ethanol-chloroform extraction would have to be avoided and column conditions would have to be chosen to handle the large amounts of Hb present.

This technique would, theoretically, not separate the desired catalase (isoelectric point (Ip) if 5.7 (22)) from other enzymic components having lower isoelectric points. In other words, even though a large amount of

undesired protein would be adsorbed to the resin, a heterogeneous, acidic mixture would obviously result. Furthermore, the procedure of adsorbing the Hb to the resin and allowing the desired enzymic component to flow through the column unretarded would limit the column load. Conditions would have to be chosen such that the large concentration of Hb would not be appreciably bound to the resin binding sites since even a slight binding by the hemoglobin could presumably have an undesired displacement effect on selectively adsorbed protein. Subsequently, the desired enzymic protein would be selectively eluted from other protein adsorbed to the resin by increasing the ionic strength.

Either the $Ca_3(PO_4)_2$ adsorbent of Tiselius (17), which displays only a slightly stronger adsorptive affinity for Hb relative to Dowex-2 (7) and selectively adsorbs high molecular-weight proteins rather than the smaller peptides and amino acids, or the cellulose resins of Sober and Peterson (18), which have large capacities for protein, would probably be more applicable systems.

The distinct advantage of the cellular resins is their hydrophilic nature in contrast to the hydrophobic properties of the Dowex and IRC-50 resins. The intermolecular forces of the aqueous buffer about the resin active site may not be symmetrically distributed about each of the molecules because of phases: the solid resin and liquid buffer phases. Under these conditions,

less stable proteins could become stereochemically modified in their continual contact with or passage through this region of dissymmetry in being bound to and released from the resin site.

In contrast, the hydrophilic nature of the cellulose resin would provide a binding site and resin backbone well saturated with buffer, even into the resin interstices. Consequently, a selective, electrostatic retardation of protein would occur in a relatively homogeneous molecular field with a greater uniformity with respect to intermolecular attraction, keeping protein denaturation due to this phenomenon to a minimum.

In considering these various theoretical and practical aspects it was decided that DEAE-cellulose would apply more favorably to the general chromatographic approach desired. The limitation in the application of this resin would be the relatively strong adsorptive affinity that this resin has for Hb (18). Hence, the allowable upper pH at which no Hb affinity for resin would occur would be considerably limited.

Catalase has been adsorbed and quantitatively eluted (from DEAE-cellulose) by gradually approaching the isoionic point of the protein by means of increasing the CO_2 pressure of the movable water phase (18). Although in specific instances (19) changes in pH have been used to elute desired protein from resins, elution may not necessarily be sensitive

to pH changes. The reason for this phenomenon will be discussed later. However, since release of multivalently bound protein is usually quite sensitive to ionic strength increase (20), elution via increase in ionic strength is more commonly practiced.

In conclusion, the chromatographic procedure should meet certain requirements. Could enzymic protein be resolved with adequate separations from the Hb present? Could pH and ionic conditions be found whereby enzymic proteins having Ip's closer to the Ip of Hb than that of acid phosphatase (Isp of 4.4) (21) such as catalase and carbonic anhydrase (Isp's of 5.7) (22) and 5.3 (23), respectively) be resolved not only from the Hb, but from each other? Lastly, what degree of purity could be attained in comparison with their crystallized counterparts?

Theoretically, ionic conditions should be found whereby in the pH range between 5.3 and 6.8 (the respective Isp's of carbonic anhydrase and Hb), Hb would theoretically pass directly through the column. Carbonic anhydrase and catalase, as well as other "acidic" enzymic protein, would be selectively eluted by an increase in ionic strength by NaCl.

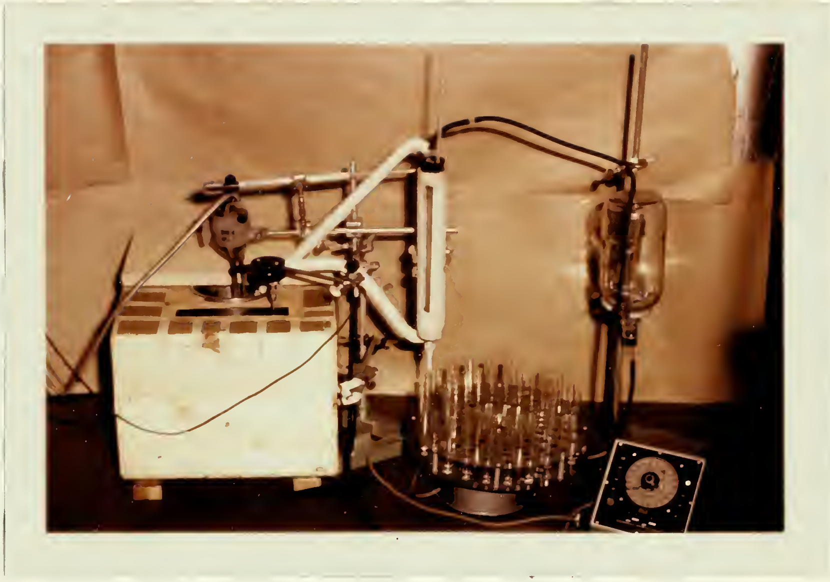
EXPERIMENTAL

APPARATUS, MATERIALS, AND METHODS

Column apparatus - The chromatography was carried out in the low temperature apparatus shown on page 9. The column (1.20 by 25 cm.) containing the resin was jacketed and its temperature was controlled by circulating water kept cold by a well insulated ice-bath. The column jacket and conducting tubes were insulated by asbestos and the resulting temperature gradient of the system was only 1.5 ± 0.5° C. Since the column could be kept at a relatively constant, low temperature and little ice was required to keep the column at this temperature over long periods of time (48 hrs.), this system proved applicable to low temperature chromatography.

Resin - DEAE-cellulose resin, obtained from Eastman Organic Chemicals, was washed by decantation with 1N NaOH, then washed with H_2O to remove the alkali. The resin was acidified to a pH of 6 with concentrated H_3PO_4, washed with H_2O, then appropriate buffer. After resin use, 1N NaOH was added to the resin to remove residual protein. The resin was washed several times by decantation with H_2O, suspended in 0.005M phosphate buffer of pH 6.60 (or other desired molarity and pH), stirred, and acidified with 0.005M NaH_2PO_4 until the pH remained constant at 6.60. The resin was then washed several times by decantation and resuspended in 0.005M buffer, pH of 6.60. The resulting slurry was evacuated at the suction pump for 30 minutes to remove air bubbles.

CHROMATOGRAPHIC APPARATUS



Column preparation - The cellulose resin, in the form of a very dilute slurry, was poured into the column until the slurry reached the top of the column. A slow flow rate was applied and as the settling of the resin proceeded, the fine suspension above the settled resin was slowly and carefully stirred at intervals to keep the settling suspension homogeneous. It should be noted that DEAE-cellulose is extremely flocculent and tends to coalesce into a "rippled pattern" if allowed to remain stationary in suspension too long or if the slurry is too concentrated during the settling process. Consequently, it is important that the settling suspension be of low concentration. Further addition of resin took place at intervals that permitted a continual settling of resin suspension during the whole length of the column preparation in order to prevent "breaks" in the resin column.

The equilibration of the resin was slow, especially after regeneration by 1.0M NaOH because of the exceedingly low molarity of the buffer used. Hence, the pH of the effluent buffer had to be closely checked with that of the incoming buffer before application of the extract.

Hemolysate preparation and application to column - Fresh, hog erythrocytes from oxalated hog blood were successively washed three times by diluting the erythrocyte suspension to two times the original blood volume with saline phosphate, pH of 7.4 (24), and centrifuging. The cells were hemolyzed by diluting the cells to three times the original blood volume

with cold water. This step sufficiently diluted the high concentration of protein present in the hemolysate before application to the column. The resulting crude hemolysate was dialyzed in the cold for 24-30 hrs. against several changes of the appropriate buffer.

The change in pH of the dialyzed hemolysate from the original pH of 7.4 to 6.6 (the pH of the equilibrated buffer) after 15 hrs. indicated that dialysis was nearing completion. Complete dialysis of the extract was important because the relatively high concentration of Cl^- from the saline would have an undesired displacement effect on enzymic protein on the resin. After completion of the dialysis, the denatured protein and stromata were removed by centrifugation. The desired amount of stroma-free supernatent was carefully applied to the side of the column in order not to disturb the column surface.

After the extract had settled into the resin at a flow rate equivalent to the flow rate during the chromatographic separation (5 ml./hr./cm.^{2} resin), the residual extract was carefully washed down the sides of the column with the appropriate buffer before the final application of the eluting buffer to the column.

ASSAYS

Catalase Assay - The method of Bonnichsen, Chance, and Theorell (25) was followed in which H_2O_2 decomposition by catalase was determined at room temperature (25°C.) at a pH of 7.0 over a short reaction period of 30 seconds by titration with permanganate.

Two ml. quantities of the assay mixture were rapidly pipetted at 10 second intervals into appropriate 15 ml. erlenmeyer flasks, which contained one c.c. of 1% H_2SO_4.

The titrimetric method is reliable for a variety of reasons (25). The H_2O_2 concentration can be kept low so as to keep enzyme inactivation due to H_2O_2 to a minimum. In addition, the activity is tested in a minimum of time (0-30 seconds) decreasing the error due to inactivation of activity upon dilution. Also, a large number of assays can be run with ease in a relatively short time, a necessity in the analysis of protein fractions in which it may be necessary to make quite a large number of determinations. The accuracy of this method has been estimated at approximately 5% (26).

A series of typical curves obtained from a purified catalase fraction of the chromatogram in figure 10 (page 38), is shown on page 13. Purified extract (0.20-0.40 ml.) was added to the assay to give the necessary half-time reaction of approximately 30 seconds. Under these conditions, H_2O_2 decomposition followed a first-order reaction curve. The standard curve was obtained using 0.10 ml. of the dialyzed hemolysate* in order to determine the total catalase activity applied to the column.

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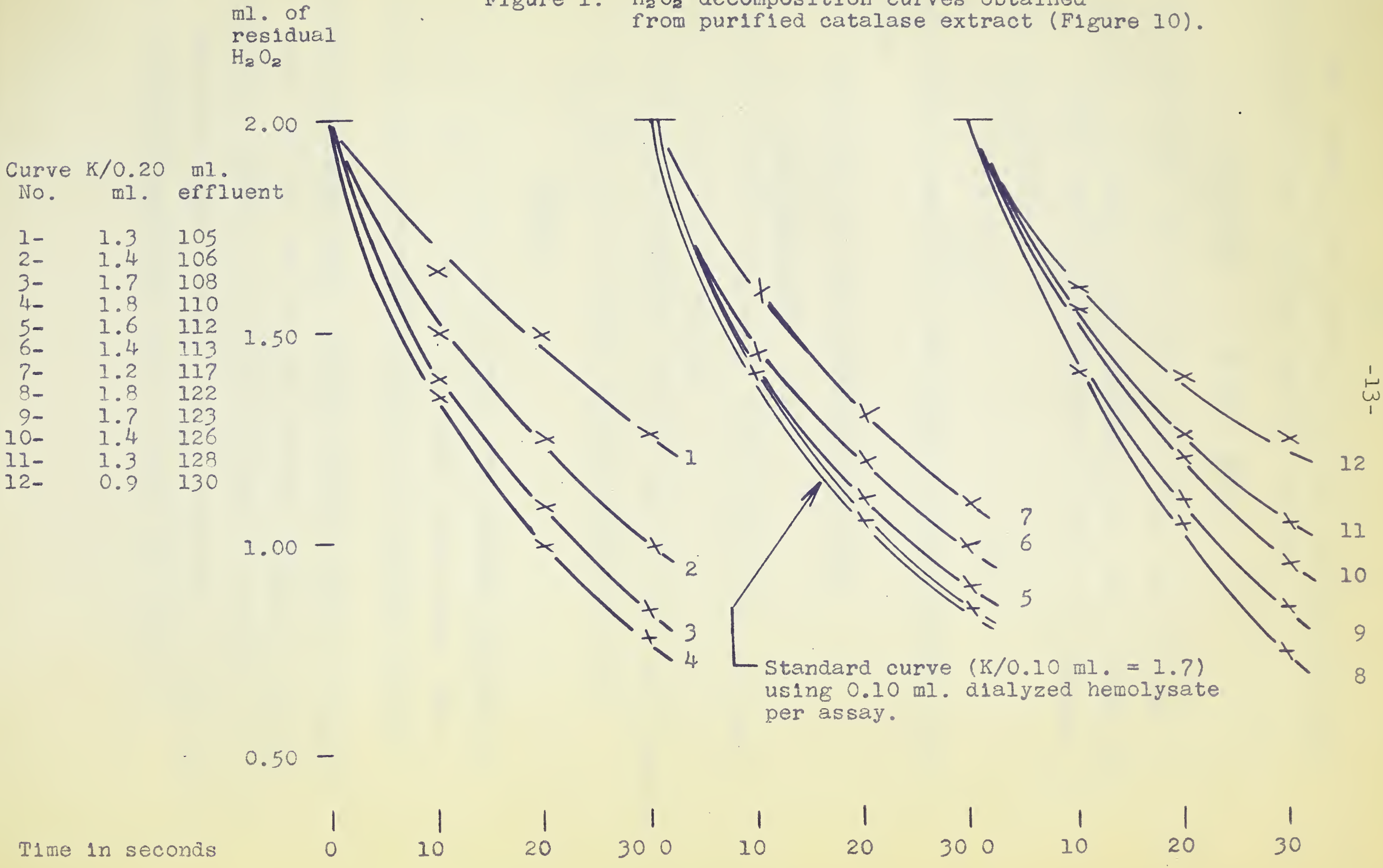
* The term "dialyzed hemolysate" refers to the extract prepared as shown on page 11, which is to be applied to the column resin.

Figure 1. H_2O_2 decomposition curves obtained from purified catalase extract (Figure 10).



Curve No.	K/0.20 ml.	ml. effluent
1-	1.3	105
2-	1.4	106
3-	1.7	108
4-	1.8	110
5-	1.6	112
6-	1.4	113
7-	1.2	117
8-	1.8	122
9-	1.7	123
10-	1.4	126
11-	1.3	128
12-	0.9	130

Standard curve (K/0.10 ml. = 1.7) using 0.10 ml. dialyzed hemolysate per assay.

The overall rate constant has been expressed in terms of a second-order reaction velocity constant:

$$K_1 = \frac{\ln\ (x_0/x)}{et} \qquad \text{Moles}^{-1}\ \text{min.}^{-1}$$

where x_0 = H_2O_2 conc. at time 0, x = H_2O_2 conc. at time t, e = conc. of enzyme (molar concentration), and t = time (25).

A pseudo first-order specific reaction rate constant (k) has been defined:

$$\underline{k} = K_1 e = \frac{\ln x_0/x}{t}$$

which gives a measure of the amount of enzyme present in the assay.

The k value has been determined by using

$$\underline{k} = \frac{2.303\ \log_{10} x_0/x}{t} \quad \text{min.}^{-1}$$

where x_0 = initial H_2O_2 conc., and x = H_2O_2 conc. at time t where t is the time in minutes.

The Katalase-fahigkeit (Kat.f.) unit of Euler and Josephson (26) has been used to express catalase purity in which

$$\text{Kat.f.} = \frac{\underline{k}}{2.303\ W}$$

where k is the pseudo first-order rate constant obtained in the test, and W is the grams of enzyme in the test. The k value has been obtained from 0.20 ml. of purified extract.

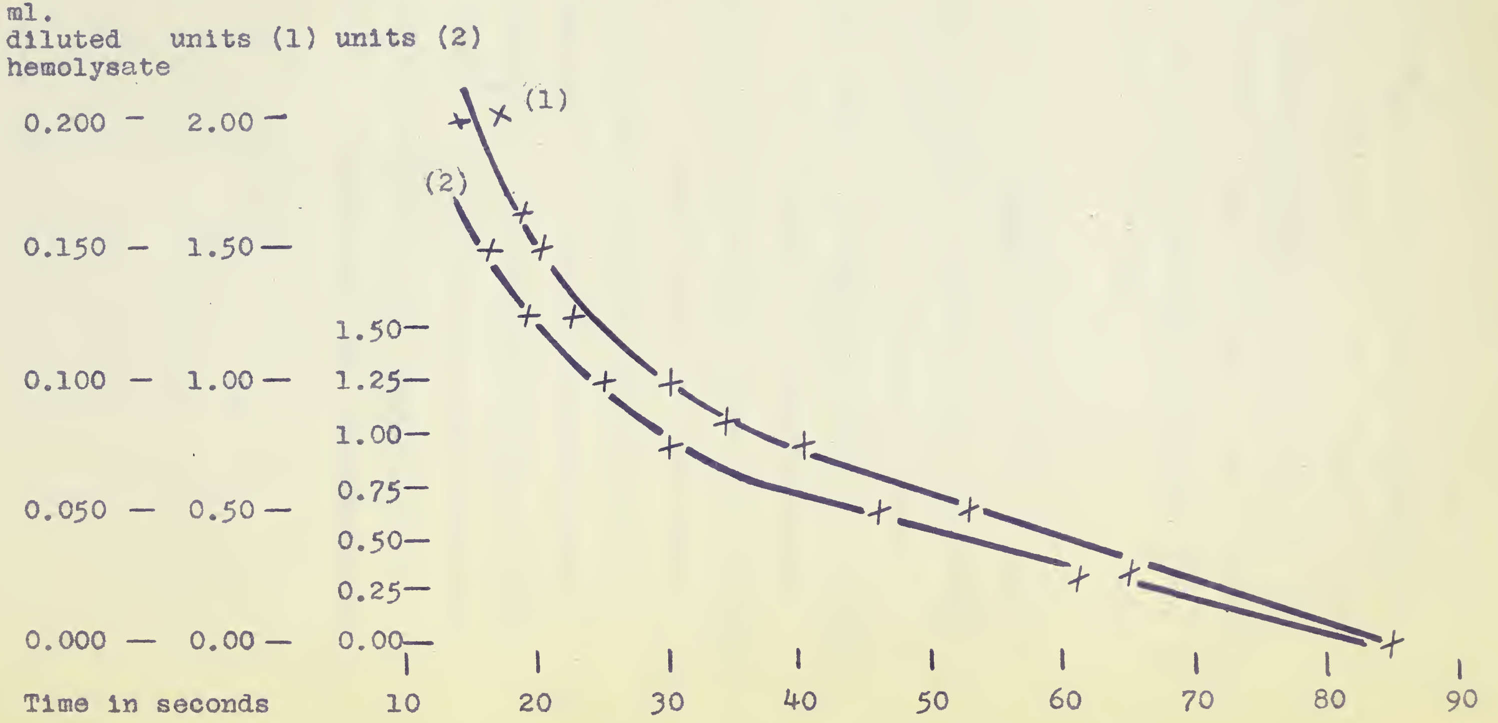
Carbonic Anhydrase Assay - The CO_2-veronal method of Roughton and Booth (27) was used to assay carbonic anhydrase activity. The rate of hydration of CO_2 was followed by measuring the time for the pH to shift from 8.15 to 6.2, indirectly, by the use of the indicator bromothymol blue, in which the change of

color from blue to yellow (at 6.5-6.2) was observed visually.

The disadvantages are: the indicator may inhibit the activity (8% by phenol red (28)) and the enzyme is subject to quite a pH variation, during which time the action of inhibitors and activators may vary (29). If the accuracy of visual determination of the end point in the range from 25-30 seconds is estimated to be 30 ± 2 seconds, the unit variation as determined from the standard curve would be 1.00 ± 0.06 units resulting in a 6% error. The pipetting error during the assay, as well as temperature variations during a series of assay determinations in which ice baths are used throughout for assay vessels and reagents, would bring the percentage error considerably higher. The overall accuracy of duplicates in this colorimetric method has been conservatively estimated at ± 10% (30). The sensitivity of the method has been increased by the determination of the end point photoelectrically (31) and recently, spectrophotometrically (30). The colorimetric method was chosen over the manometric method (32), which although accurate and quantitative is limited by the complexity of the manipulations necessary for the measurement of a large number of samples in a short period of time.

The standard curves on page 16 were obtained from the original, dialyzed hemolysate (page 11) using a dilution of 0.10 ml. dialyzed hemolysate/4.0 ml. of water. Curves no. 1 and no. 2 were obtained from hemolysates used for the chromatograms of figures 9 and 10, respectively. Appropriate

Figure 2. Standard curve for carbonic anhydrase assay using a dilution of 0.10 ml. dialyzed hemolysate/ 4.0 ml. H_2O.



volumes (0.0125-0.050 ml.) from the various fractions collected were used in the assay to give a reaction time in the region of 30-50 seconds from which the respective carbonic anhydrase units were read directly from the graph. Enzymic activity has been expressed in units/0.025 ml. or units/0.05 ml. in which one enzyme unit has been arbitrarily defined as the amount of enzyme required to shorten the blank time of 85 seconds to 30 seconds.

Protein Analysis - Protein fractions from the column were followed by the method developed by Warburg and Christian (33), in which the ultraviolet (U.V.) absorption was determined in a Beckman model DU spectrophotometer at 280 mμ. This U.V. absorption is due primarily to the presence of tyrosine and tryptophane and since these amino acid contents are considered to vary only slightly with various enzymes, it has been considered relatively accurate. Some variation, however, has been found with the smaller molecular weight proteins of the pancreas (34). The method was well suited for chromatographic analysis of the protein fractions since it is rapid, extremely sensitive to small quantities of protein, and the presence of salts does not interfere with the determinations.

RESULTS

PRELIMINARY EXPERIMENTATION

Preliminary experimentation involved the application of purified catalase extract to small columns (1.0 by 15 cm.) at room temperature in order to determine the pH and ionic conditions of catalase adsorption and elution. From previous work in the literature, it was learned that ionic strength conditions for protein adsorption on the cellulose resins had to be considerably lower than that for IRC-50, in which columns were usually equilibrated in the range of 0.2-0.3M buffer. Hence, ionic conditions for DEAE-cellulose were arbitrarily set at 0.050M phosphate buffer.

At a pH of 5.7 (Ip of catalase), catalase activity flowed quantitatively through the column, but at pH's of 6.3 and 6.8, only 60-65% of the activity moving with the "buffer front" could be accounted for. The remaining activity, however, was eluted by the addition of NaCl. The pH had to be raised to 7.2 before catalase was completely adsorbed and at this pH up to a pH of 8.0, catalase was quantitatively eluted (0.30M NaCl).

The next problem involved determining if catalase activity could be adsorbed onto the resin at a lower pH by lowering the ionic strength of the equilibrating buffer, since it seemed quite probable that Hb would be adsorbed at

this pH of 7.2. Three column runs were made at lower ionic concentrations at a pH of 6.8. At a phosphate buffer concentration of 0.030M, catalase was adsorbed to the extent of 70%, but at 0.015 and 0.005M, catalase was completely adsorbed and quantitatively eluted (0.30M NaCl).

An overall summary of the above results is shown below.

pH	BUFFER CONC.	% ADSORPTION
5.7	0.050M	0%
6.3	0.050M	35%
6.8	0.050M	35%
	0.030M	70%
	0.015M	100%
	0.005M	100%
7.2	0.050M	100%

From the results of this preliminary investigation, it was concluded that catalase activity is quantitatively adsorbed at about 0.02M, and is eluted in the range of 0.07-0.1M phosphate buffer at a pH of 6.8.

A large scale experiment followed in order to find out if these pH and ionic conditions would apply to crude hemolysates. In setting up the experiment, it was evident that a cold temperature apparatus would have to be used because under all of the column conditions described above, a certain portion of the Hb was being irreversibly bound to the resin,

probably due to the denaturation of the Hb at room temperature. The apparatus used has been described on page 8.

The resin was equilibrated at a low ionic strength (0.005M) and the eluting buffer of 0.02M was applied, stepwise, to find out how much protein could be eluted from the column before the final release of catalase activity (0.020M + 0.06M NaCl).

A column load of 4 ml. of dialyzed hemolysate (a dilution factor of 4 times the original blood volume was used rather than the 3-fold dilution indicated on page 11) was arbitrarily chosen and applied to the column (34 c.c. of equilibrated resin).

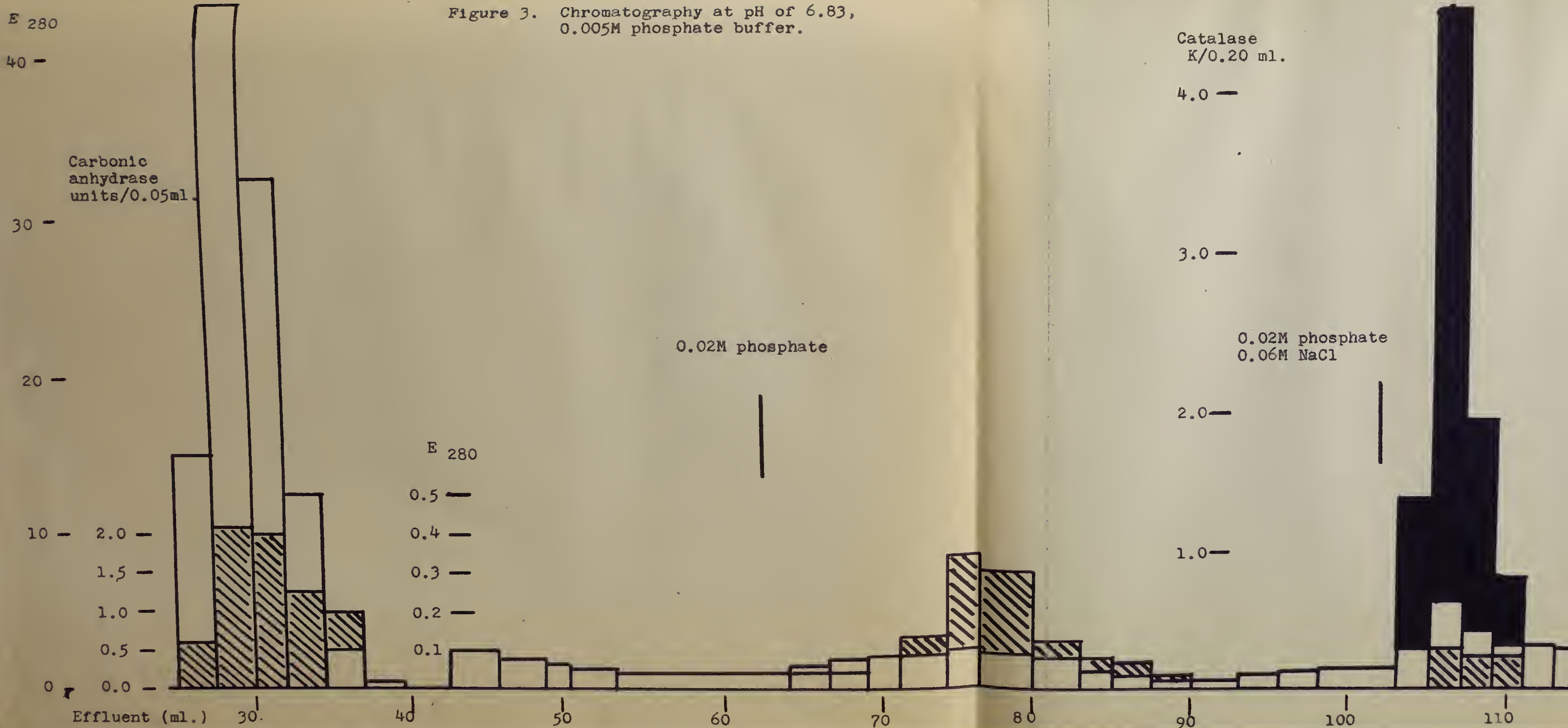
As shown in figure 3, only a relatively small amount of protein could be eluted from the resin by increasing the eluting medium to 0.02M phosphate after the emergence from the column of the "breakthrough" peak. This small fraction of protein was shown to contain 30% of the carbonic anhydrase activity applied to the column. Upon the analysis of further fractions, however, it was found that another 30% of the activity was emerging with the "breakthrough" peak and a small amount of residual activity was appearing in the catalase fraction. Hence, it was apparent that under the pH and ionic conditions chosen, the resin was not selectively adsorbing this activity from the hemolysate to any marked degree.

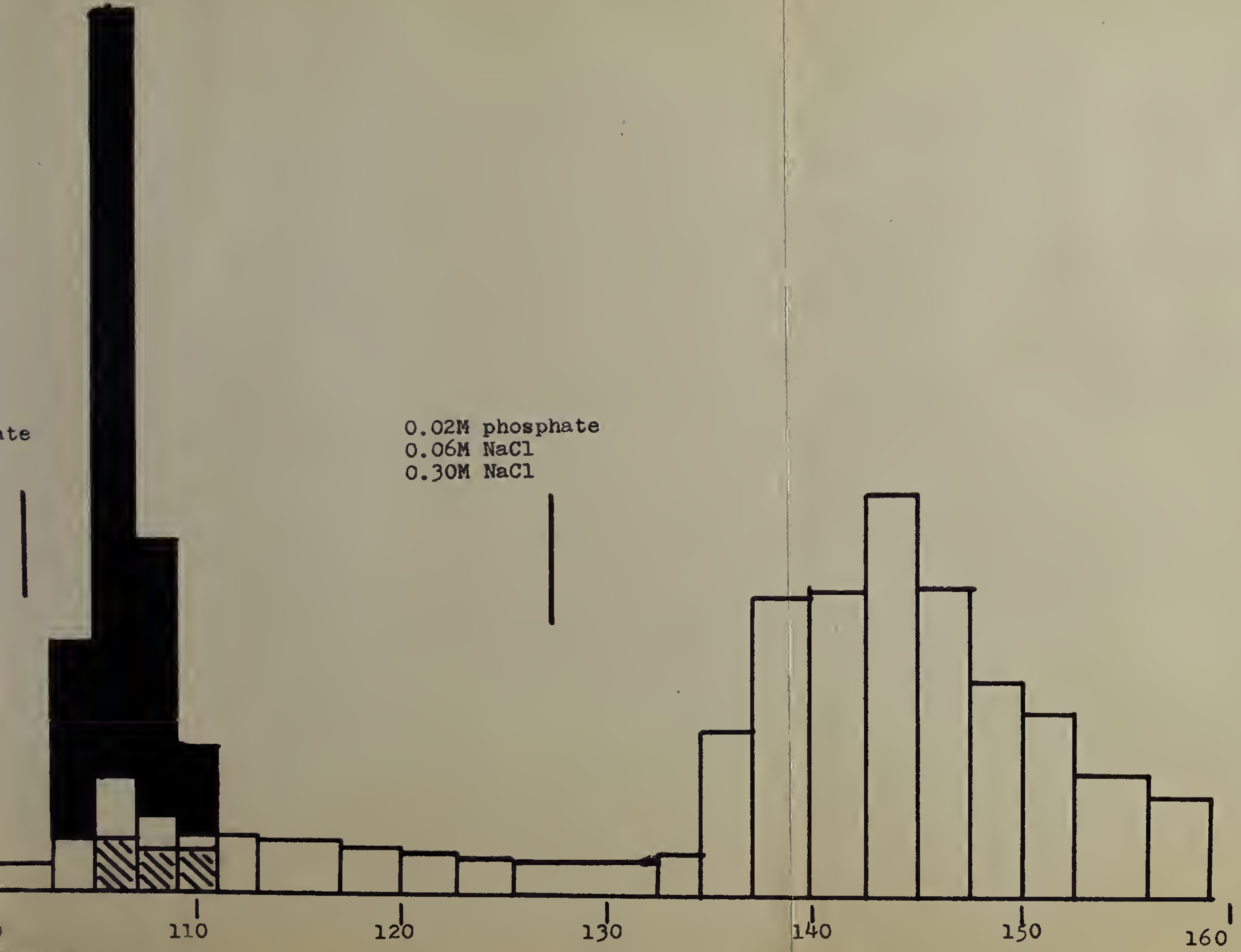
In the following chromatograms, protein carbonic anhydrase and catalase activities, and hemoglobin have been represented as shown below.

PROTEIN (Extinction at 280 mu:)	▭
CARBONIC ANHYDRASE ACTIVITY	▨
CATALASE ACTIVITY	■
HEMOGLOBIN (Extinction at 540 mu.)	═

The eluting buffer steps have been placed in the chromatograms at the point of actual ionic step increase.

Figure 3. Chromatography at pH of 6.83, 0.005M phosphate buffer.




ate
0.02M phosphate
0.06M NaCl
0.30M NaCl
110
120
130
140
150
160

Also, elution of the residual, adsorbed activity was not very sensitive to increased ionic strength (a 4-fold increase from the original equilibrated buffer was required to separate most of the carbonic anhydrase activity from the resin).

These activities show a somewhat unique behavior in that carbonic anhydrase, although a relatively more "acidic" protein, completely precedes the catalase fraction. An extensive discussion of this behavior will be given later.

In considering the column load, if 1 extinction unit is approximately equivalent to a protein concentration of 1 mg./ml. (the approximate relationship obtained for the purified yeast enolase of Warburg and Christian (33)), the total adsorbed protein that could be eluted after the emergence of the "breakthrough" peak totalled about 25 mg. or 2/3 mg. protein per 1 ml. equilibrated resin. Hence, the load used would approach the adsorptive capacity of the Amberlite IRC-50, and Dowex-2 resins (35).

In conclusion, the chromatogram shows that a further, relatively large amount of protein can be eluted from the resin using 0.30M NaCl.

A STUDY OF CATALASE AND CARBONIC ANHYDRASE BEHAVIOR UNDER VARIOUS CONDITIONS OF IONIC STRENGTH AND PH.

The following series of experiments were set up to determine whether or not 0.02M phosphate buffer was the maximum ionic strength which could be applied to the column to remove residual protein and carbonic anhydrase (C.A.) activity before the final elution of catalase.

In addition, a series of runs at various pH's would have to be made in order to determine if C.A. activity could be resolved to a greater extent from the protein of the "breakthrough" peak. The upper pH limit would be confined to the pH at which Hb would become quite strongly bound to the resin.

Procedure - The following experiments were carried out in a cold room at 4°C. on small columns (1.0 by 12 cm. containing 11 c.c. of equilibrated resin). A column load of 2 ml. of dialyzed hemolysate was applied.

Results - At pH values of 6.83 and 7.2, 0.005M phosphate, (figures 4 and 5, respectively) in which the stepwise gradient has been slightly increased from the previous value of 0.02M (figure 3) to 0.03M, a portion of the catalase activity has emerged from the column, the elution being somewhat more pronounced at the pH of 6.83. The

eluting concentration has had to be increased (0.06M NaCl) in order to elute the remaining residual activity quantitatively. The increase in pH from 6.8-7.2 has somewhat increased the capacity of the resin for catalase in that a relatively greater fraction of the residual activity was eluted at pH of 7.20. The increase in pH to this upper limit (Hb was beginning to bind more strongly to the resin) did not increase the resin capacity for C.A. activity, however.

Improvement in the resolution of C.A. activity was attempted by lowering the buffer strength from 0.005M to 0.0025M. As shown in figures 6, 7, and 8, a large portion of the activity was still contained in the "breakthrough" peak despite the lower ionic strength and the higher pH's (up to 7.6). The amount of residual protein (mostly Hb) bound to the resin increased, as the pH was increased as indicated by the relatively greater amount of protein eluted (0.02M) in the C.A. fraction of figure 8 (Extinction at 280 mμ = 5 units) in contrast to the protein eluted in figure 6 ($E_{280} = 0.15$). In addition, at pH of 7.6, the "breakthrough" peak had begun to spread over a greater effluent volume, the extinction of the most concentrated fraction decreasing from an E_{280} value of 60-65 at pH's of 6.8 and 7.2 to an E_{280} value of 25.

Conclusions - From this series of chromatograms, it was shown that 0.02M phosphate is the maximum buffer concentration which can be applied stepwise to elute residual protein with carbonic anhydrase activity before the final elution of catalase.

Since the ionic strengths for catalase adsorption and elution (figure 4) were within relatively narrow limits, it was decided that an ionic gradient could be applied to resolve this activity. In direct comparison, an 8-fold increase in the original buffer concentration, added stepwise, (figure 6) was required to elute the remaining C.A. activity completely from the column.

An increase in the pH of the equilibrating buffer did not improve the resolution of C.A. activity. In fact, if the areas of C.A. activity are compared in figures 6, 7, and 8, the chromatogram of figure 6 (pH of 6.63, 0.0025M) has given the best quantitative resolution of the activity with the least amount of residual protein in the fraction. Hence, these pH and ionic conditions have been used for the attempted large scale resolution in the following section.

Figure 4. Chromatography at pH of 6.83, 0.005M phosphate buffer.

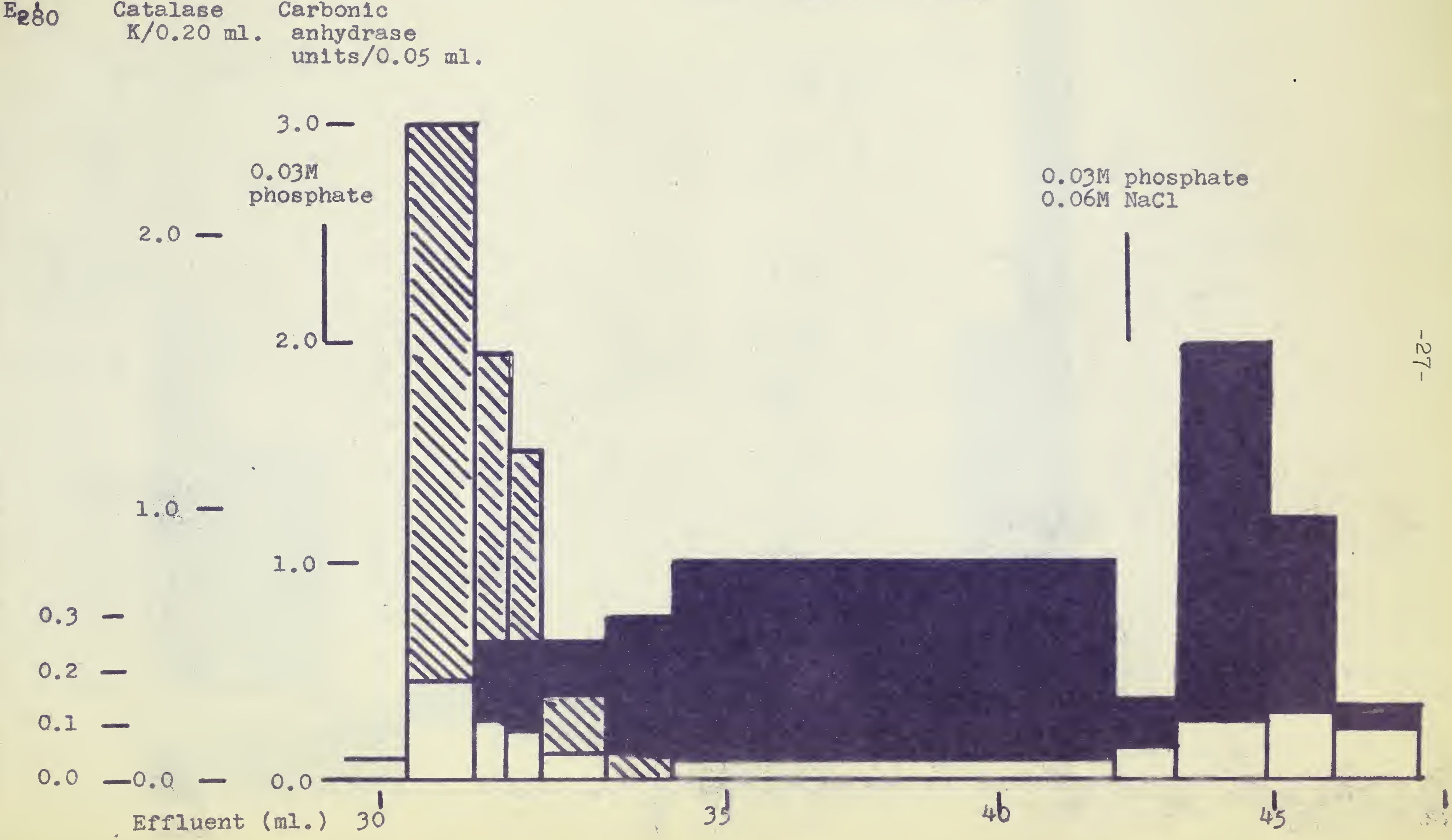


Figure 5. Chromatography at pH of 7.20, 0.005M phosphate buffer.

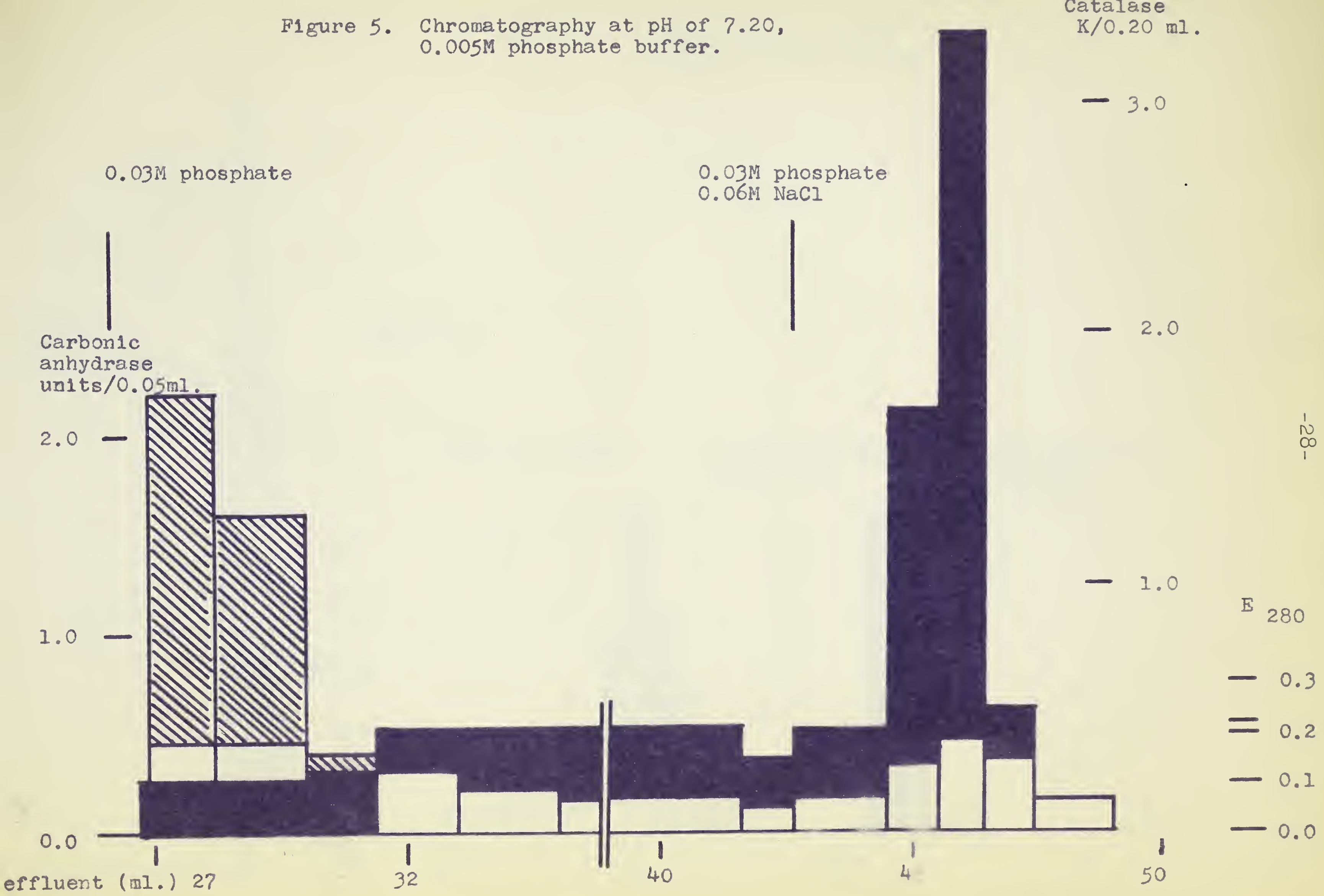


Figure 6. Chromatography at pH of 6.62, 0.0025M phosphate buffer.

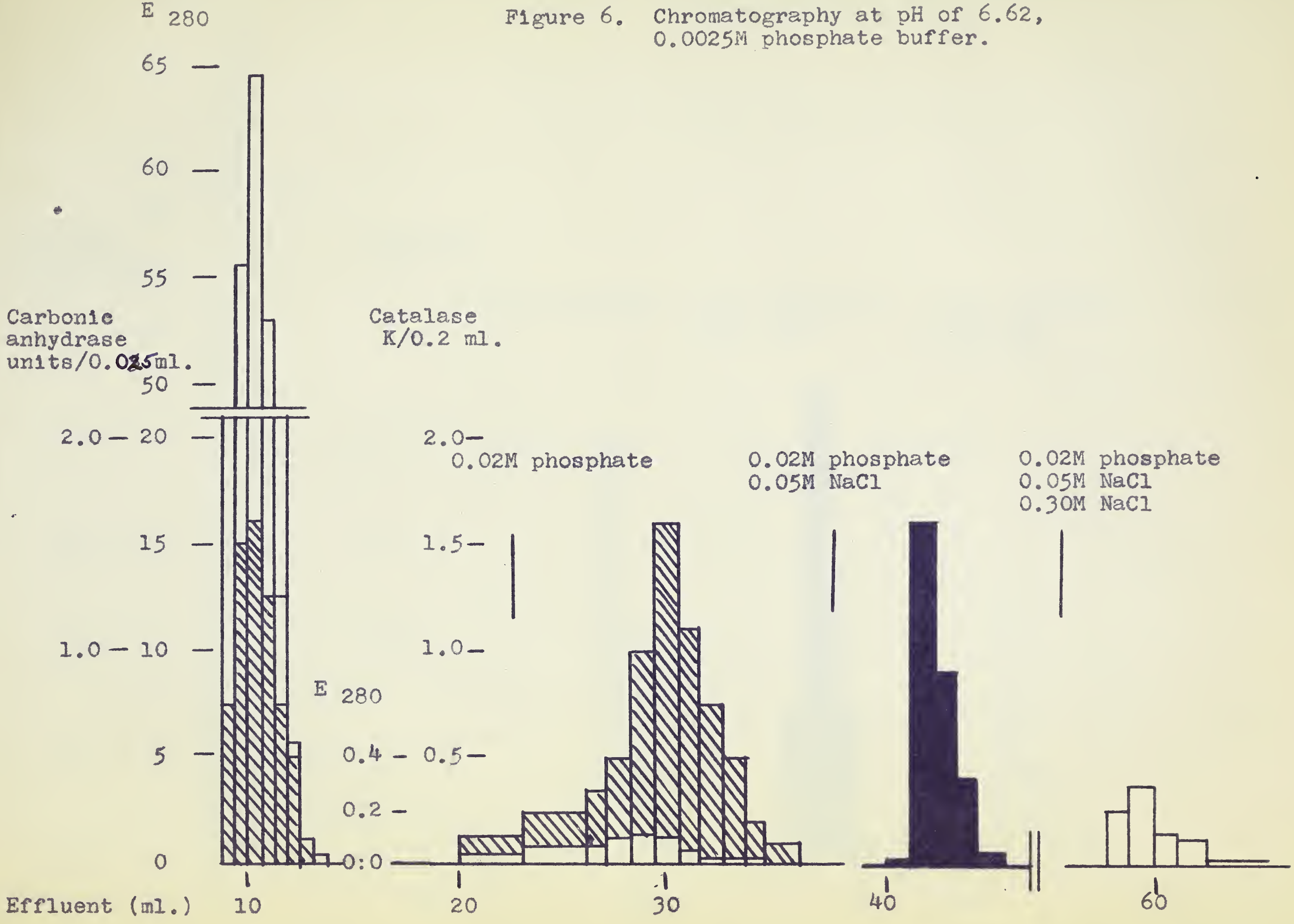


Figure 7. Chromatography at pH 7.05, 0.0025M phosphate buffer.

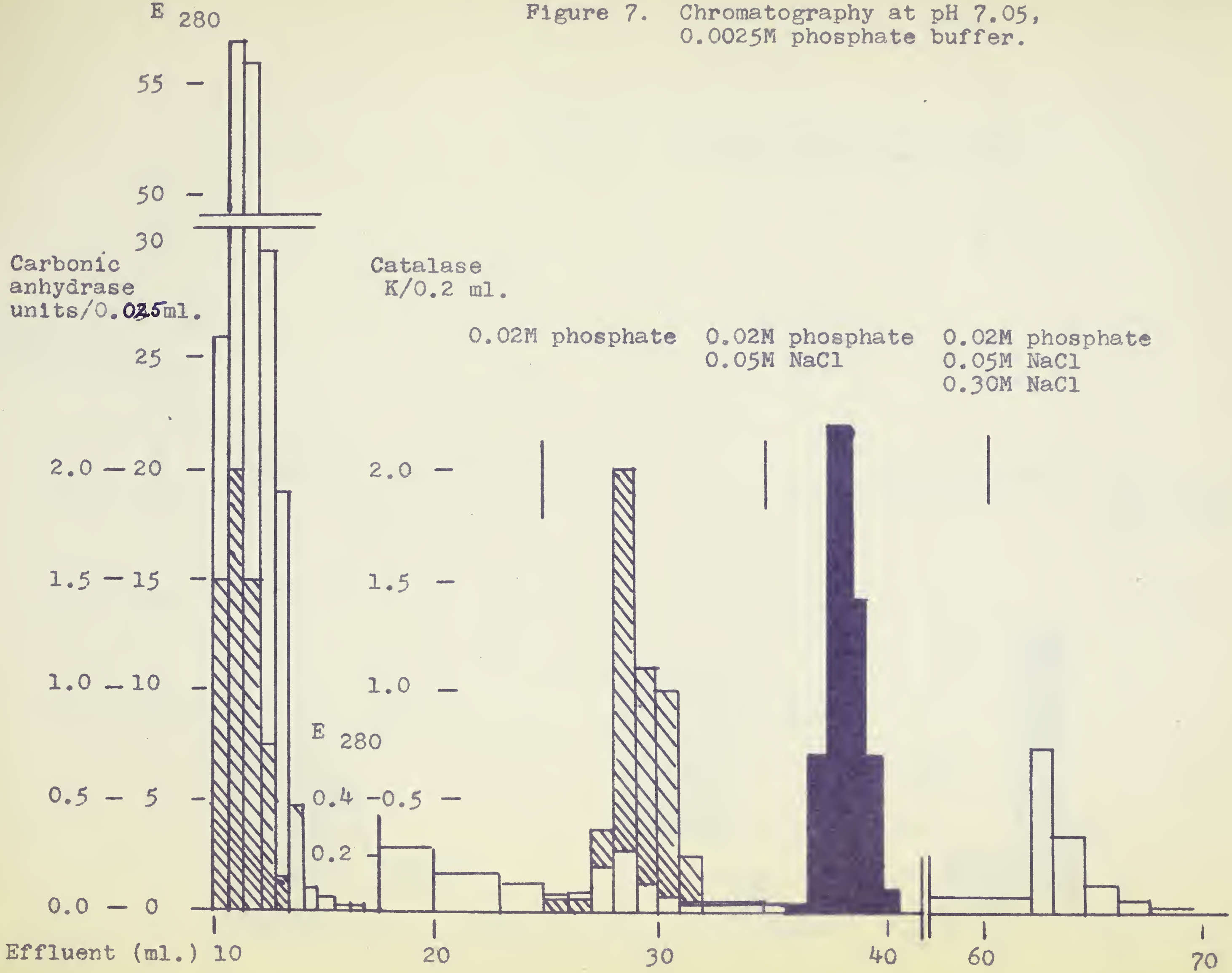
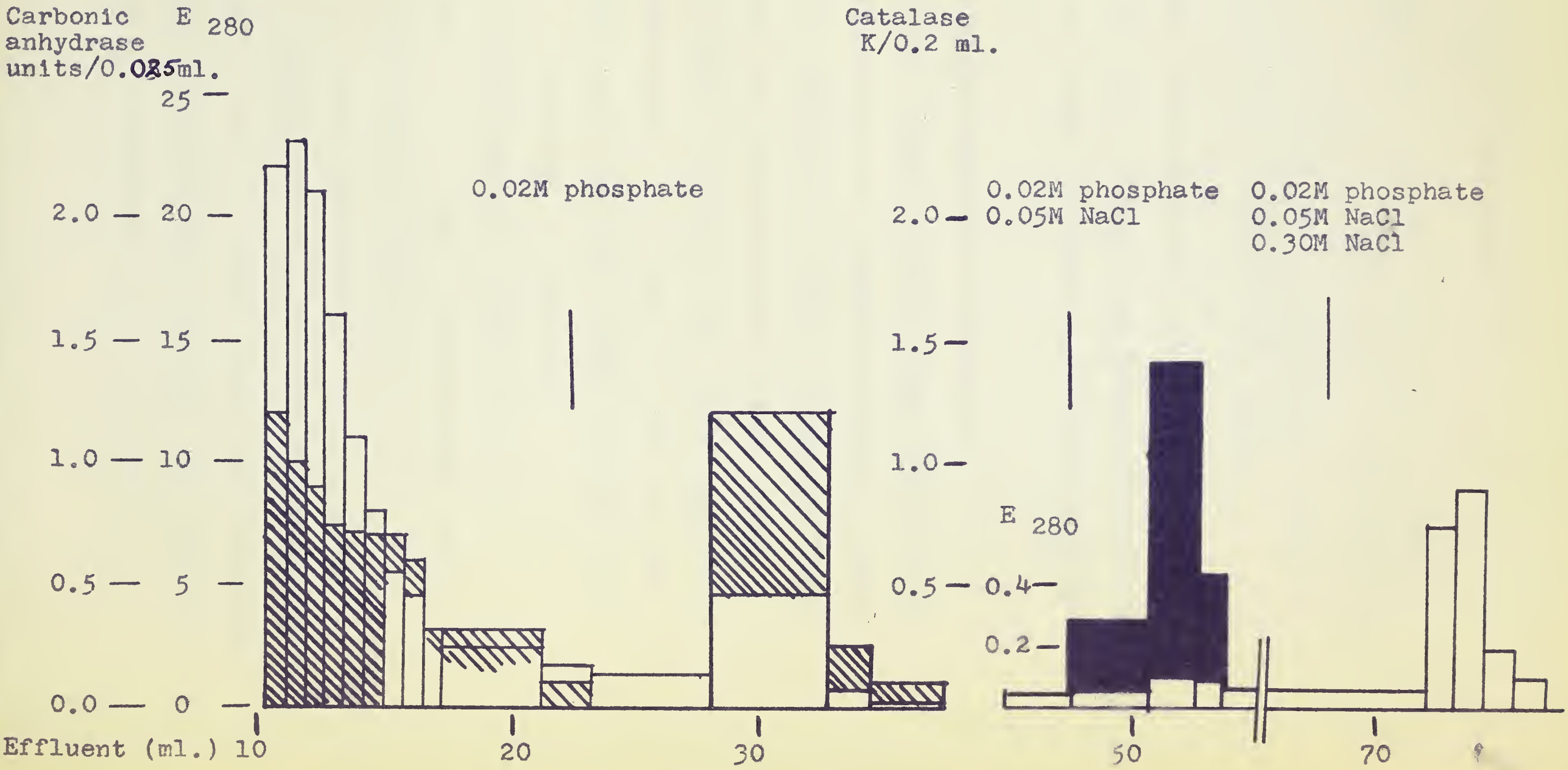


Figure 8. Chromatography at pH of 7.60, 0.0025M phosphate buffer.



LARGE SCALE EXPERIMENTATION

Having determined the optimum conditions for catalase and C.A. adsorption and elution, large scale experimentation was carried out with the large, low temperature column (page 8) using comparatively large column loads.

Procedure - The resin was equilibrated with 0.0025M phosphate, pH of 6.62, and a stepwise gradient of 0.02M was used to elute C.A. activity, followed by an ionic gradient to resolve catalase.

The gradient elution apparatus consisted of a 250 ml. erlenmeyer flask as the "common" mixing vessel (containing 0.02M phosphate buffer mixed by a magnetic stirrer), which was fed by inflowing buffer from a 500 ml. dropping funnel (containing 0.50M NaCl in 0.02M buffer in figure 9 and 1.00M NaCl in 0.02M buffer in figure 10). Under the above conditions, the salt conc. in figure 9 would increase from 0.02M to 0.075M over an effluent range of 25 ml. and to 0.15M per 25 ml. of effluent in figure 10.

The column load was increased 4-fold (12 ml. of dialyzed hemolysate/34 c.c. of equilibrated resin) over that used in the chromatogram of figure 3.

The dialyzed hemolysate and catalase fractions were collected, dialyzed exhaustively against distilled water to remove the last traces of buffer salts, taken down to dryness at 90°C and immediately weighed upon cooling. The specific activity of catalase was determined on a dry weight basis.

Results - The elution patterns of two column runs are shown in figures 9 and 10. It is evident from figure 9 that C.A. activity has spread over an extremely wide effluent range. Two main fractions of activity, the first contained in the "breakthrough" peak and the second relatively free of Hb, emerged from the column at a constant ionic strength of 0.0025M. The stepwise application of 0.02M phosphate eluted two further bands of activity, the first closely associated with a small amount of residual Hb, which emerged within the leading edge of the step, the second, free of Hb but accounting for only 10% of the activity applied to the column. The total of the four fractions accounted for 50-60% of the total activity. Catalase emerged, via the gradient, free of C.A. activity over an effluent volume of 35 ml. and could be accounted for quantitatively. The same general results have been obtained in figure 10, except that a larger amount of residual Hb containing a comparatively large portion of activity has been eluted at 0.02M. Twice the ionic gradient concentration used in figure 9 has eluted catalase within a narrower effluent volume. The elution of two further protein fractions, via the gradient, is quite evident in figure 10, but no attempts have been made to correlate these fractions with enzyme activity.

The degree of purity of the two purified fractions must be discussed. On a dry protein weight basis, the catalase fractions of figures 9 and 10 have given extinction values of

13.7 and 13.2, respectively (based on 1 per cent solutions measured at 280 mμ). It should be noted that these extinction values were based on results obtained using a total of only 1 mg. dry weight protein. Hence, weighing errors (± 0.2 mg.) and failure to quantitatively remove such small amounts of protein from the dialysis bags could contribute a significant error to these values (an extinction value for carbonic anhydrase could not be determined because of the excessively low amounts of protein available). Nevertheless, the above values were found to compare favorably with the value of 14.7, obtained for bovine liver catalase.* Hence, the following catalase purities have been based on the values of 13.4, which would make 1 extinction unit equivalent to 0.75 mg. protein/ml. extract.

A sample calculation of purity (from figure 10) is given below. The k value (pseudo first-order constant) was obtained for a specific volume of extract (0.10-0.20 ml.). Since k is directly proportional to the enzyme concentration, 1 catalase unit has been arbitrarily defined as the amount of enzyme necessary to give a k value of 1 min.^{-1}. From the standard curve (page 13), 0.10 ml. of dialyzed hemolysate gave a first-order reaction constant of 1.7 min.^{-1} (1.7 catalase units/0.10 ml.). Hence, 12 ml. of dialyzed

*quoted by Frank R.W. Gurd, in Protein Methods and Procedures.

hemolysate applied to the column would contain 200 units per 12 ml. of hemolysate or 0.28 units/mg. protein.[*] Using average values of 1.5 min.$^{-1}$ for k and 0.15 for the extinction, the enzyme could be quantitatively accounted for in a total of 3.0 mg. of protein resulting in 67 units/mg. protein. Comparing the values obtained for the crude and purified fractions, a 240-fold increase in purification had been attained with a resulting Kat.f. of 29,000.

Since Kat.f. values have been defined for a temperature of 0°C., the value of 29,000, obtained at room temperature, had to be corrected accordingly. Chance and Herbert (36), by using a rapid titrimetric method, have shown that the Arrhenius equation relating the first-order velocity constant and absolute temperature held true over the temperature range of 0°-30°C. Using the energy of activation for blood catalase as 1700 calories/mole (37), the corresponding Kat.f. at 0° was calculated to be 23,000.

The chromatogram of figure 9 has given essentially the same purification (a 235-fold increase in purification with a Kat.f. of 22,000).

In considering the C.A. activity, if 0.0025 ml. of dialyzed hemolysate contained 1 unit of activity (curve no. 2, page 16), a column load of this hemolysate would contain 4800 units with a specific activity of 6.7 units per

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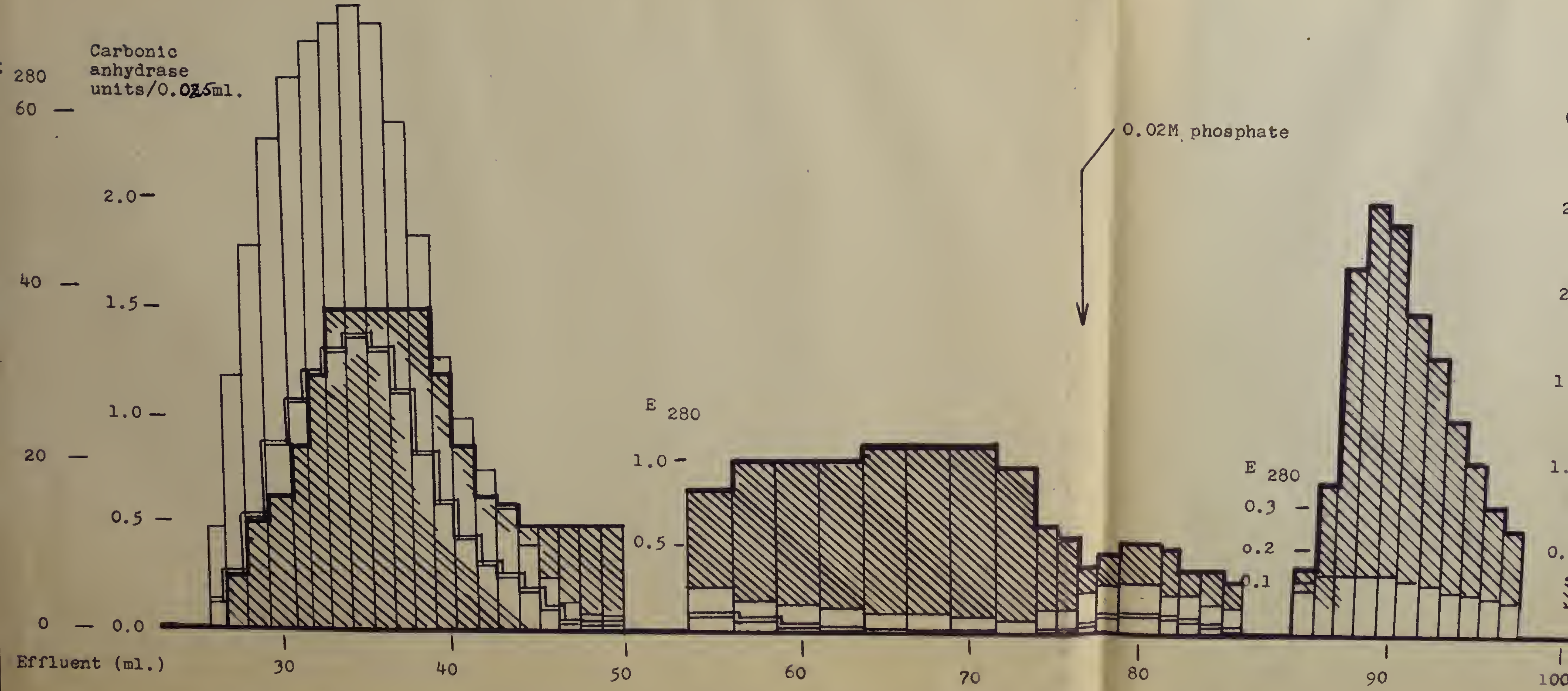
[*] based on the value of 60 mg. dried protein/ml. dialyzed hemolysate.

mg. protein. By using an average value of 1.5 units/0.025 ml. and an extinction value of 0.13 over the effluent range of 87-95 ml. (figure 9) of the purified fraction, 500 units of C.A. activity were accounted for with a specific activity of 630 units/mg. protein (based on the same extinction-protein relation as for catalase). Hence, an approximate 95-fold increase in purification was attained with a resulting 10% yield in this fraction.

Conclusions - The purity of this C.A. compared with the value of a 160-fold increase in specific activity (10% yield) for the crystalline preparation (38) would indicate that the preparation from the column approached two-thirds its purity.

If the Kat.f. value for hog catalase is considered to be within reasonable limits for that obtained for beef catalase (48,000 (39)), the resulting Kat.f. of 23,000 and quantitative yield would suggest that an extensive and significant purification had been attained on this resin. In addition, since this purified fraction was shown to be free of carbonic anhydrase activity, there is an indication that a high degree of resolution has been obtained.

Figure 9. Chromatography at pH of 6.61, 0.0025M phosphate buffer.



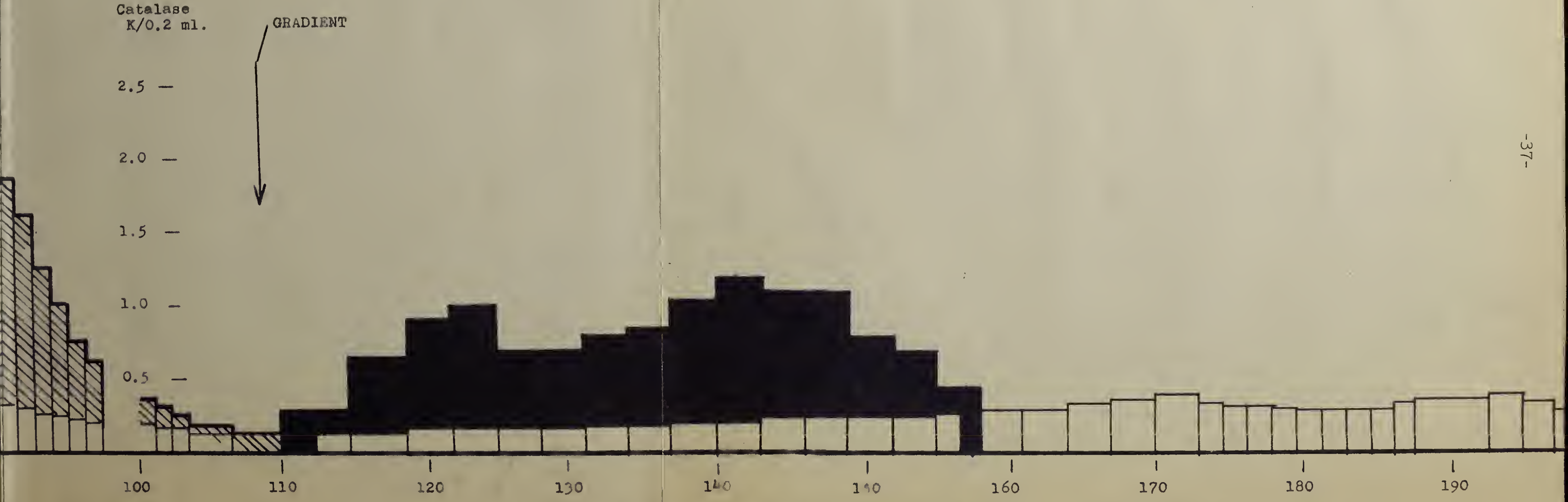
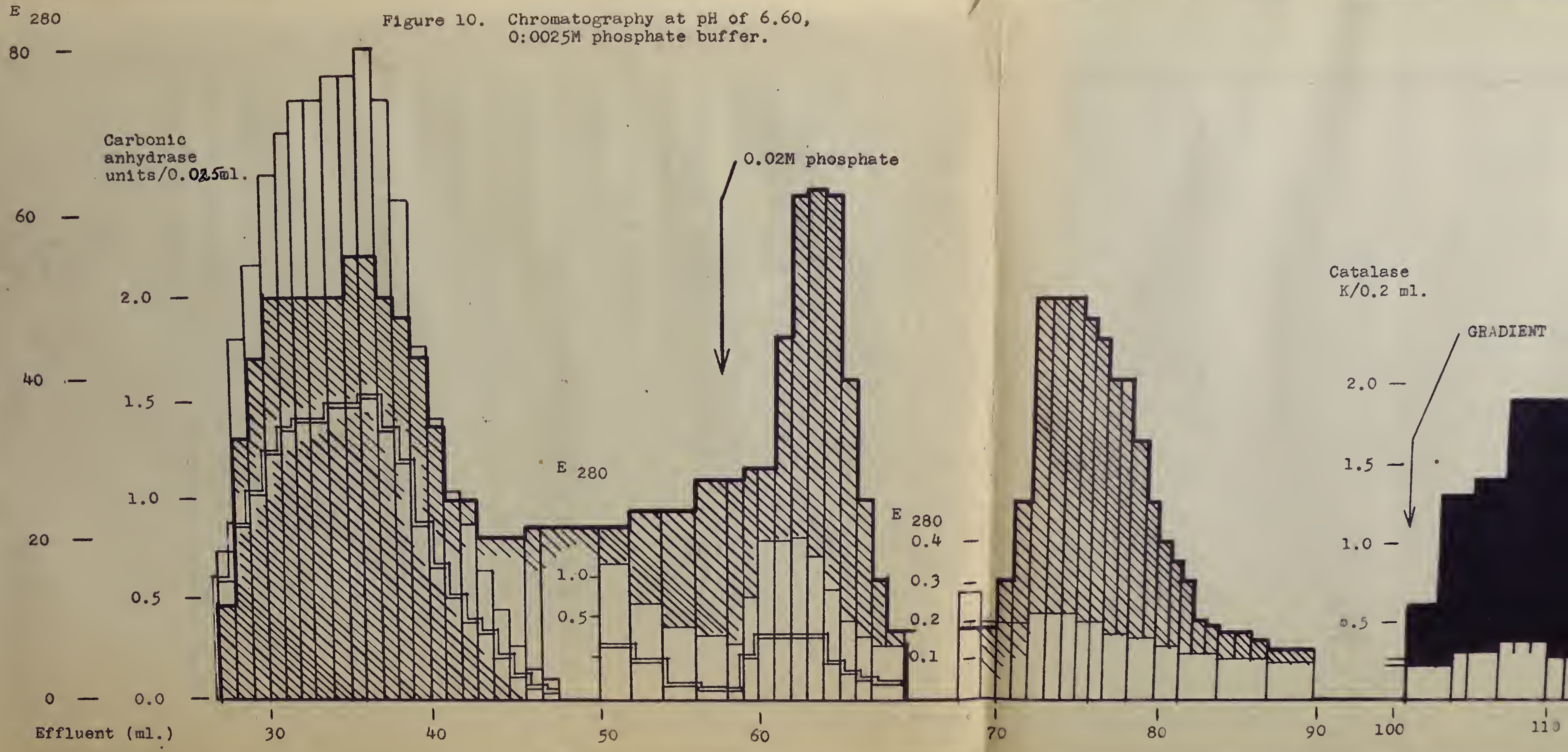
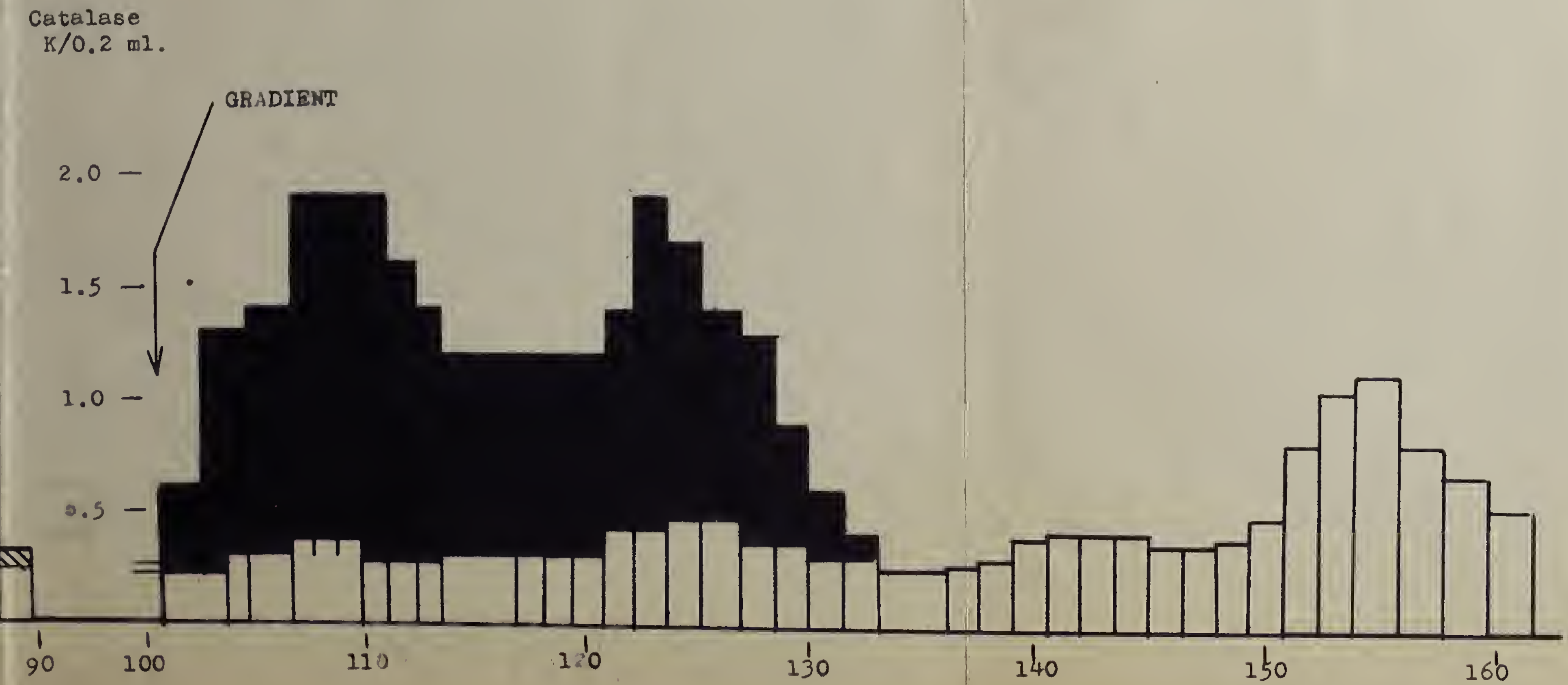

Catalase
K/0.2 ml.
GRADIENT
2.5
2.0
1.5
1.0
0.5
100
110
120
130
140
150
160
170
180
190

Figure 10. Chromatography at pH of 6.60,
0:0025M phosphate buffer.




Catalase
K/0.2 ml.
GRADIENT
2.0
1.5
1.0
0.5
90
100
110
120
130
140
150
160

DISCUSSION

It has been generally considered that if the mechanism of protein resolution on cellulose resins (18) involves long range coulombic forces between the protein and resin binding site, the order of elution of a series of proteins should show a correlation with their electrophoretic mobilities. The unique feature shown in the preceding chromatograms (figures 4, 5, and 6) was that C.A. activity unexpectedly preceded catalase, yet C.A. has a greater net negative charge and would be expected to be more tightly bound than catalase. If the spatial orientation of the amino acid side chains in the protein is considered, it is possible that specific charged groups that donate to the total net charge of the protein are folded into the interior or the molecule and, hence, stereochemically hindered from coming into contact with the resin binding site. Consequently, the distribution of charges and not merely the total net charge, may be the more important factor in the success or failure to resolve a specific protein.

Increases in pH to as high as 7.6 (figure 6) did not markedly affect the degree of resolution of C.A. activity from the hemolysate. This behavior may be accounted for, in part, by the fact that within the relatively narrow pH range

considered, the total net coulombic charge of the protein may not be changing to any great extent since the pH change may not be taking place in the buffering region of the active groups mainly responsible for the change in net charge. Other factors which may be responsible for the above behavior will be considered in the following section (page 42).

The elution pattern of catalase activity in figure 4 shows the presence of a "false" zone of activity brought about by applying a relatively lower ionic strength than that required to elute the activity completely. This phenomenon may be explained on the basis that proteins generally do not exhibit finite, reversible distributions between resin and buffer phases. Because of the availability of several charged sites on the protein for binding purposes, the protein will tend to bind irreversibly (polyvalently) to the charged resin surface. At low ionic strengths, the cellulose resin will have a relatively large number of active sites available for binding purposes and, consequently, its adsorptive capacity will be at a maximum. Under these conditions, the protein will be adsorbed to the resin in the form of a tight concentration band at the top of the column. If a small concentration step of eluting ion (Cl^-) is applied, however, the eluting ion will more strongly compete with the protein for the charged site: the net result being fewer active resin sites available for

binding the specific activity. In other words, since the resin capacity for protein has been decreased, a certain fraction of the band will migrate downward, reaching fresh resin and readsorbing again. If a further step is applied, the capacity of the resin for the protein will diminish to the extent that the protein band will spread until it saturates the entire column length. Further successive ionic steps will each release a small proportion of the applied activity, appearing in the effluent fractions as a series of "false" zones until a point is reached at which the eluting ion is of sufficiently high concentration that the resin has no longer any capacity for the specific protein.

The various zones of C.A. activity displayed in figures 9 and 10 are probably not the result of different protein entities, but more probably due to one specific activity emerging from the column as a series of "false" zones. If the second and fourth zones are considered, they would display a behavior analagous to that of catalase in figure 4. Under the ionic and pH conditions applied, the DEAE-cellulose resin would have a low capacity for C.A. activity, resulting in the emergence of a large, broad band of activity, only slightly retarded, immediately following the "breakthrough" peak. A comparatively small fraction of the total activity would be irreversibly adsorbed by the resin under these conditions (released by the step) accounting for the activity in the fourth band.

Two zones (the third and fourth of figure 9) emerge from the column under a constant ionic strength of 0.02M, the third band within the leading edge of the step apparently very closely associated with a small amount of residual Hb. Likewise, in figure 10, C.A. activity emerged at the front of the buffer step, but as a comparatively high concentrated fraction of activity associated with a correspondingly high amount of residual Hb. If some form of interaction between carbonic anhydrase and Hb were assumed, Hb could act as a "carrier" bringing about the abnormally quick elution of the small portion of activity (upon its release, the trace of residual Hb moves unretarded, emerging with the leading edge of the buffer step).

The first zone of activity may also be considered to be one of the "multiple" fractions in that the above phenomenon just described could also account for the emergence of this fraction of the activity within the "breakthrough" peak. If no such interaction were present, the activity of the first peak would be expected to appear entirely in the slightly retarded fraction no. 2. Furthermore, rechromatography of a portion of the first peak showed resolution of another fraction of activity from the Hb. Hence, this activity behaved similarily to the activity present in the original extract.

The reasons for using a linear gradient to elute catalase will be discussed. In figure 3, a stepwise gradient (0.06M NaCl) was used to release catalase, in which the activity appeared, highly concentrated, over quite a narrow effluent range. If the elution strength necessary to elute the specific activity quantitatively is not known, and an excessively large step is applied, further protein of a heterogeneous nature may be released. With the application of the linear gradient, however, the most efficient elution range necessary to release the desired protein will always be met. Since catalase release was found to be quite sensitive to increased ionic strength, the use of a gradual ionic gradient seemed more desirable than the use of a step. By knowing the approximate ionic limits of catalase adsorption and elution, the degree of gradient necessary for its elution could be predetermined.

The artifact involving the separation of two zones of catalase activity may be attributed to the gradient apparatus used, in which a long tygon tube carried the buffer from the reservoir (dropping funnel) to the mixing chamber. If at the beginning of the gradient application, the buffer levels in both chambers were of the same level, the flow of buffer from the reservoir would not begin until a sufficient pressure head had been developed (to overcome friction within the narrow tube) by the lowering of the

level in the mixing chamber. Once the buffer being fed into the mixing chamber began to move, however, it would keep flowing until the levels again became almost even. Hence, the resulting gradient would begin more sharply than normal, eluting a portion of the activity as a sharp front, finally leveling off to the normal gradient.

In summary, it is apparent from these preceding results that regardless of how general a chromatographic method may appear in theory, it may certainly prove somewhat limited in its application. The reverse in elution order and the marked difference in resin capacity (resulting in the comparatively non-specific separation of C.A. from the Hb) for the two enzymic proteins was totally unexpected.

The nature of the stereochemistry of the charged amino acid side chains could certainly play a part in this somewhat unique behavior in that, with respect to catalase, the charged sites responsible for the net charge of the protein would be readily available for electrostatic binding with the resin. The adsorptive force must certainly be predominantly coulombic in nature to account for the ease of release via the increased ionic strength (20).

With respect to C.A. on the other hand, the active charged groups may be sterically hindered from interacting with the resin and the slight retardation of activity attained may be predominantly due to "secondary" irreversible forces

since, comparatively speaking, the release of this activity was so insensitive to ionic strength increase.

If a higher pH limit could be imposed upon the system, C.A. activity could undoubtedly be quantitatively adsorbed and eluted with some degree of selectivity, but the unique feature in the chromatography of R.B.C. hemolysates is that the pH range applied to resolve any specific activity may be somewhat limited due to the presence of Hb. With respect to DEAE-cellulose, for example, the adsorptive capacity for Hb appeared quite strong, a portion being bound tightly to the top of the column at a pH slightly acidic to the Isp. Consequently, this factor could seriously limit the upper pH range which may be necessary to adsorb other desired protein components at low ionic strengths. The answer to this specific problem may lie in the application of the hydroxyapatite columns of Tiseleus, in which it has been noted that Hb binds to a comparatively lesser degree (17).

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